Postprinmt of: Environmental Geochemistry and Health (2020) https://doi.org/10.1007/s10653-020-00625-z

Strategies in a metallophyte species to cope with manganese excess

Rossini-Oliva, S[1]*, Abreu, M.M[2]., Leidi, E.O[3]

[1]Department of Plant Biology and Ecology, University of Seville, Av.da Reina Mercedes, POB 1095, 41080 Seville, Spain

[2]Linking Landscape, Environment, Agriculture and Food Research Centre (LEAF), Instituto Superior de Agronomia, Universidade de Lisboa, Lisboa, Portugal

[3]Department of Plant Biotechnology, IRNAS-CSIC, Av.da Reina Mercedes 10, 41012 Seville, Spain

ORCID Rossini-Oliva, S.: 0000-0001-6774-4723

ORCID Abreu, M.M.: 0000-0002-6650-1161

ORCID Leidi, E.O.: 0000-0003-3425-2030

Abstract

The effect of exposure to high Mn concentration was studied in a metallophyte species, *Erica andevalensis*, using hydroponic cultures with a range of Mn concentrations (0.06, 100, 300, 500 and 700 mg L^{-1}). At harvest, biomass production, element uptake, and biochemical indicators of metal stress (leaf pigments, organic acids, amino acids, phenols, and activities of catalase, peroxidase, superoxide dismutase) were determined in leaves and roots. Increasing Mn concentrations led to a decrease in biomass accumulation and tip leaves chlorosis was the only toxicity symptom detected. In a similar way, photosynthetic pigments (chlorophylls *a* and *b*, and carotenoids) were affected by high Mn levels. Among organic acids, malate and oxalate contents in roots showed a significant increase at the highest Mn concentration while in leaves, Mn led to an increasing trend in citrate and malate contents. An increase of Mn also induced an increase of superoxide dismutase activity in roots and catalase activity in leaves. As well, significant changes in free amino acids were induced by Mn concentrations higher than 300 mg L^{-1}, especially in roots. No significant changes in phenolic compounds

were observed in the leaves but root phenolics were significantly increased by increasing Mn concentrations in treatments. When Fe supply was increased 10 and 20 times (7-14 mg Fe L^{-1} as Fe-EDDHA) in the nutrient solutions at the highest Mn concentration (700 mg Mn L^{-1}), it led to significant increases in photosynthetic pigments and biomass accumulation. Manganese was mostly accumulated in the roots and the species was essentially a Mn excluder. However, considering the high leaf Mn concentration recorded without toxicity symptoms, *E. andevalensis* might be rated as a Mn tolerant species.

Keywords: *Erica andevalensis*, manganese, metallophytes, uptake, organic acids, amino acids, catalase, peroxidase, superoxide dismutase

Introduction

Acid soils usually contain excessive levels of potentially toxic elements like Mn and Al, and many plants species have developed adaptation strategies to survive and thrive in such limiting environments (Marschner 1995). In the particular case of very acid soils contaminated by mining activities (e.g. the Pyrite Belt in the Iberian Peninsula) hold high concentration of other phytotoxic metals and metalloids like As, Cu, Fe, Pb and Zn (Abreu et al. 2008; Márquez-García and Córdoba 2010; Monaci et al. 2011). The location of some plant communities along the banks of highly acid and contaminated rivers exposes the species to periodical floods and waterlogging which may increase even more the availability of toxic metals (Rodríguez et al. 2007; Abreu et al. 2008; Marschner 1995). This scenario is the natural habitat of a metallophyte species, *Erica andevalensis* Cabezudo & Rivera, which thrives on these soils and accumulates and tolerates Mn even where the metal is not present at high concentration (Abreu et al. 2008; Monaci et al. 2011; Rossini Oliva et al. 2018). *Erica andevalensis* is a vulnerable and endemic species of the Iberian Pyrite Belt (Cabezudo and Rivera, 1980), able to colonize successfully mine tailings allowing a vegetative cover (Rossini-Oliva et al., 2018, Pérez-López et al. 2014).

Manganese is an essential element for plants but an excessive accumulation may produce toxicity. Many plant species show dark spots on leaves, crinckled leaves as main toxicity symptoms (Foy et al. 1978; Fernando and Lynch 2015) and greater activity of enzymes related to metabolism of reactive oxygen species generated by Mn toxicity (Leidi et al. 1987, 1989; Fecht-Christoffers et al. 2006; Millaleo et al. 2010). Manganese is oxidased to Mn^{3+} in the cell wall by peroxidases producing typical symptoms (brown spots) and leaf injuries (Fecht-Christoffers et al. 2006). In shoots of Mn-hyperaccumulator plants (able to accumulate more than 10,000 mg kg^{-1}), the metal

is accumulated at very high concentration without toxicity symptoms through efficient systems of metal compartmentation (Krämer 2010). In some species like *Acanthopanax sciadophylloides* and *Phytolacca spp*, most Mn appears complexed with oxalate (Memon and Yatazawa 1984; Dou et al. 2009; Xu et al. 2009).

Non-hyperaccumulator plants have developed different adaptation mechanisms to cope with high Mn concentrations such as limited transport into shoots by root fixation or compartmentation in root vacuoles, chelation and storage in leaf cell vacuoles or leaf structures (glands, trichomes) to avoid the Mn-induced generation of toxic oxygen radicals in the cell wall and the cytosol (Horiguchi 1987; Ernst et al. 1992; Reichman 2002; Sharma et al. 2016). The increased synthesis of carboxylates has been related to metal chelation and its vacuole storage (Pittman 2005) and malate and citrate are mostly the organic acids associated with Mn in the vacuoles (Führs et al. 2012; Blamey et al. 2015). The induction by high Mn concentration of some tonoplast metal transporters, like the cation diffusion facilitator or metal transporter proteins (MTP8) or other less specific metal transporters (CAX) might be responsible for Mn vacuolar accumulation (Migocka et al. 2014; Sharma et al. 2016). Meanwhile, available Fe may effectively reduce Mn uptake and toxicity symptoms (Marschner, 1995). The aim of this study was to determine tolerance to high Mn in *Erica andevalensis* by answering questions like how much Mn can tolerate the species? Or which are the main organic chelators induced by metal excess? How some reactive oxygen scavengers react to Mn toxicity?

Materials and Methods

Plant culture

Seeds of *Erica andevalensis* were collected in Peña de Hierro (Riotinto mining area, SW Spain) during Spring. The seeds were sterilised in 0.3 % hypochlorite and

95 washed three times with sterile distilled water, placed to germinate in Petri dishes on a
96 double layer of filter paper. After germination, seedlings were transferred into tubes
97 filled with rockwool and 8 L plastic buckets with a nutrient solution (pH 4.0) reported
98 by Rossini-Oliva et al. (2012) at 1/10th strength. When seedlings were approximately 4–
99 5 cm height, the experiment was started by adding different concentrations of Mn (100,
100 300, 500, and 700 mg L^{-1}) as $MnSO_4$ to the nutrient solution. The Mn concentrations
101 were chosen considering that the available Mn concentration found in soils of S.
102 Domingo mine (Portugal) was approximately 100 mg L^{-1}, and to test the Mn tolerance
103 of this species we multiplied it by a factor of 3, 5 and 7. The basic nutrient solution
104 (control) contained 0.06 mg L^{-1} Mn. The solutions were continuously aerated with an
105 aquarium air pump, and renewed every seven days to maintain a constant nutrient
106 supply and metal concentration. The experiment was carried out in a growth chamber
107 with cycles of 26–22 ºC (day-night temperature) and 16 h light/8 h darkness. In order to
108 study the effect of Fe on Mn stress alleviation, plants were cultivated in a nutrient
109 solution containing the highest Mn concentration reported above (700 mg L^{-1}) and 7 or
110 14 mg L^{-1} of Fe as Fe-EDDHA. These concentrations were chosen according to
111 previous lab studies. The experiment was carried out during 45 days and plants were
112 weighed at 15–days intervals. All treatments had four replicates and eight plants in each
113 replicate.
114
115 ***Plant analysis and growth measurement***
116 At harveste, plants were separated into leaves and roots and washed with
117 distilled water. Four plants per replicate were oven-dried at 70 ºC during 48 h and dry
118 biomass of shoots and roots was determined. The remaining plants were sampled for
119 biochemical assays and the remainings frozen and liophylized for further organic acids

analyses. Oven-dried plant material was milled and digested with a HNO_3 in a Digiprep digestor. Elements concentration (B, Ca, Cu, Fe, K, Mg, Mn, P, S and Zn) in roots and shoots was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). Elements concentrations were determined by the method of standard additions and were expressed in mg element per kg dry weight. Procedural blanks were usually below the detection limit. Biomass production was calculated as the difference between the fresh weight at the beginning and the end of the experiment. Water content (WC) in roots and shoots was calculated at harvest as:

$$WC = [(\text{fresh weight} - \text{dry weight})/\text{fresh weight}] \times 100.$$

The ratio between shoots and roots dry biomass was also calculated.

Translocation coefficient (TC), the quantitative ratio between Mn concentration in plant leaves and roots was calculated to recognize the preferential partitioning of Mn to the aerial part (TC values>1).

Determination of photosynthetic pigments, organic acids and phenolic compounds

Photosynthetic pigments were determined in methanolic extracts obtained from shoot tips after extraction for 24 h in darkness at room temperature according to Lichtentahler (1987). The analysis of organic acids in shoots and roots was performed by HPLC for identifying main carboxylates. Then, quantification was performed using enzymatic kits (L-malic acid, citric acid and Enzytec oxalic acid, R-Biopharm). For HPLC separation, a Synergi Hydro-RP column and 20 mM KH_2PO_4 pH 2.9 (eluent) were used. Peaks were detected with a PDA detector (Waters 2996). The concentration of phenolic compounds was determined in ethanolic extracts from shoots and roots

using the Folin-Ciocalteu reagent and the concentration was estimated using a standard curve of chlorogenic acid (Chirinos et al. 2007). The analyses were run in triplicate.

Catalase, peroxidase, and superoxide dismutase activities

Shoots and roots were frozen in liquid N_2 and ground with mortar and pestle. Then, tissue samples, three per treatment (approx. 0.1 g fresh weight) were homogenized with plastic rods in Eppendorf tubes with 50 mM Tris-HCl buffer pH 7.5, containing 0.1 mM EDTA, 2 mM dithiothreitol and 0.2% Triton X-100 (1:20, weight:volume ratio). After centrifugation at 10,000 *g* (4 ºC, 15 min) enzymes activities were determined in the supernatants. Catalase activity was determined in crude extracts following decrease in A_{240nm} at 20 ºC in phosphate buffer 50 mM pH 7.0 containing 15 mM H_2O_2 (Aebi 1984). Peroxidase was assayed following pyrogallol oxidation at A_{420nm} in phosphate buffer 25 mM pH 6.0 containing 0.025% H_2O_2 (Jiménez et al. 1997). A photochemical assay (Giannopolities and Ries, 1977), was used to determine superoxide dismutase activity with methionine, riboflavin and *p*-nitro blue tetrazolium (NBT) measuring inhibition of NBT photoreduction at A_{560nm} (25 ºC). Protein in the supernatants was determined with Bradford's reagent (Bradford 1976).

Free amino acids in leaf and roots

For the extraction of free amino acids from shoots and roots, three frozen samples maintained at -70 ºC were homogenized in 80% ethanol by crushing tissues with plastic rods, set in ultrasonic bath for 5 min, centrifuged and filtered through 45 µm membranes. Amino acids were separated and quantified after derivatization with phenylisothiocyanate by reverse-phase high-performance liquid chromatography

(Heinrikson and Meredith 1984) with a Waters chromatographic system (Water 510 pumps, 717 autosampler, absorbance detector 486, and Pico.Tag column).

Statistical analyses

Data were tested for normality by Shapiro-Wilk test ($p > 0.05$) prior to conducting the analyses. Differences of variables (elements concentration, biomass, water content, photosynthetic pigments, etc.) among treatments and plant parts were tested using the ANOVA method, followed by the Tukey post-hoc multiple comparison test. For variables that were not normal nonparametric test of Kruskal-Wallis was applied to compare multiple independent samples and Mann-Whitney U test was also used to test differences between two groups. A correlation analysis (Pearson) was performed between the Mn concentration in nutrient solution and the other elements in the different plant parts and physiological parameters. All the statistical analyses were performed by Statistica (StatSoft Inc., USA) software program and probability level was set to $p < 0.05$.

Results

Plant growth and physiological parameters

Chlorotic leaves appeared when plants were grown in aqueous solutions with 500 or 700 mg Mn L^{-1} (Figure 1). However, no dark dots or spots, typical leaf symptoms of Mn toxicity in many plant species, were detected. Plants treated with 300 mg Mn L^{-1} or higher Mn concentration showed a slight growth reduction at the beginning but growth resumed the following weeks (Fig. 2). Biomass accumulation was affected by the Mn treatments ($p=0.001$) (Fig. 3), with a continuous inhibition at each Mn increase in the nutrient solution. Significant differences in the plant biomass were

observed between all Mn treatments compared with the control, but no differences were observed between 100 and 300 mg Mn L^{-1} or between 300 and 500 mg Mn L^{-1}. A negative correlation ($r=-0.40$, $p<0.05$) was found between Mn concentration in solution and plant biomass. Shoot and root water contents did not change with Mn treatments (Table 1), but the shoot/root ratio showed significant differences between Mn treatments ($p=0.001$). Shoots showed greater sensitivity than roots to the highest Mn concentration in the nutrient solution in comparison with the control treatment (73% vs 54% inhibition). Correlation analysis showed a negative correlation ($r=-0.56$, $p<0.05$) between Mn concentration and plant shoot/root ratio.

The increase in Mn supply affected negatively the content of photosynthetic pigments ($p<0.001$) (Table 1). Chlorophylls and carotenoids concentration decreased when Mn concentration in the nutrient solution reached 300 mg L^{-1} (Table 1). When additional Fe was added to the solution with the highest Mn concentration (700 mg Mn L^{-1}), a correction in the chlorosis was observed with the corresponding increase in leaf pigments (Table 1, Fig. 1b) and a significant increase of biomass production was also observed (Fig. 3). A positive correlation was found between Fe and chlorophyll a ($r=0.55$) and b ($r= 0.57$). Both Fe treatments (7 and 14 mg L^{-1}) also affected carotenoids and chlorophyll content leading to a significant increase ($p<0.05$) in their concentration (Table 1). At high Mn concentration an increased concentration of phenolic compounds in roots was observed but not in leaves (Table 2). Increasing Mn concentration induced changes in enzymatic scavenging systems of reactive oxygen species such as a significant increase of catalase activity (CAT) in leaves and superoxide dismutase activity (SOD) in roots (Table 3). However, no significant change in peroxidase activity was found either in shoots or roots. When increasing Fe supply at the highest Mn

concentration, it led to a reduction in CAT in leaves and roots but an increase in root SOD activity (Table 3).

Among carboxylates found in roots and leaves (Table 4), clear differences were found between plant organs. In the leaves, citrate was significantly increased by Mn in the medium ($r=0.89$, $p<0.05$) while in roots oxalate (and fumarate although at low concentration recorded by HPLC analysis, data not reported) was significantly correlated with Mn concentration in solution ($r=0.94$, $p<0.05$). Malate content was also significantly increased by Mn in both leaves and roots ($r=0.94$, $p<0.001$ and $r=0.70$, $p<0.05$, respectively). High Mn in the medium also induced an increased accumulation of amino acids in roots (Table 5) particularly in aspartate, glutamate, arginine, and the amides asparagine and glutamine. In the leaves, only arginine was significantly increased by Mn (Table 5) meanwhile it was noteworthy the reduction in methionine concentration detected.

Plant chemical composition

The variation in elements concentration in leaves and roots in plants treated with Mn are presented in Table 6 and Figure 4. In the roots, the concentration of all elements was modified by Mn treatments ($p>0.05$) with the exception of S and Ca (Table 6). Root P concentration significantly increased when an additional supply of Fe (14 mg Fe L^{-1}) was provided at the highest Mn concentration (Table 6) and a similar pattern was observed for root Fe concentration (Figure 4a). Manganese supply did not change substantially root Fe concentration but it significantly diminished leaves Fe content (Figure 4b), and a significant negative association was found between Mn treatments and leaf Fe concentration ($r=-0.78$, $p>0.05$). At high Mn, supply of additional Fe led to leaves Fe concentration recovery to levels found at 100 and 300 mg Mn L^{-1} (Figure 4b).

Manganese concentration in roots increased with Mn supply but no significant differences between treatments were found except with the control. The addition of Fe at the highest Mn concentration had no effect on Mn accumulation in roots (Table 6). A competitive or antagonistic effect of Mn treatments was found in the root contents of K and Mg, which were always lower than the control when increasing Mn concentration (Table 6). Concentration of Cu in roots was not affected by Mn but it increased when plants were treated with additional Fe. A significant negative association was found among Mn root concentration with root concentration of K (r=-0.73) and Mg (r=-0.78). Meanwhile, Fe contents in roots were positively associated with root contents of Ca (r=0.45), Cu (r=0.85) and P (r=0.57).

In the leaves, increasing Mn significantly decreased the concentration of Ca, Mg, Cu, and Fe (Table 6, Fig. 4b) and negative correlation was found between Mn with Ca (r=-0.78), Cu (r=-0.80), Fe (r=-0.65) and Mg (r=-0.84). However, Mn supply did not affect the concentration of P and S in leaves. The leaf Mn concentration increased until Mn concentration reached 300 mg L^{-1} (Table 6). Interestingly, additional Fe did not reduce Mn accumulation as it might be expected (antagonism) but was positively associated with Ca (r=0.66), Cu (r=0.64) and Mg (r=0.68) contents.

The elements accumulation pattern was different between roots and leaves (Figure 5). The roots were the recipient of most Cu, P and Mn in comparison with shoots (TC<1) whilst the leaves accumulated more B, Ca, K, Mg and S than the roots (TC>1). It is interesting to note that for Fe values of TC were higher than unity for control, 100 and 300 mg Mn L^{-1} but it decreased when Mn in the nutrient solution increased and when Fe was supplied.

Discussion

Erica andevalensis, even though is a non-hyperaccumulator *stricto senso* species, as it only reached a maximum of 3,619 mg Mn kg^{-1} in leaves (Table 6), was able to tolerate up to 700 mg Mn L^{-1} (or 15 mM) in the root medium. Leaves displayed just tip chlorosis (from 500 mg Mn L^{-1}) as unique visual toxicity symptom but biomass production was inhibited even by 100 mg Mn L^{-1} (Fig. 3). Most of the uptake Mn remained immobilized in the roots (TC<1) blocking its transfer into the leaves probably either oxidized and fixed in root cell walls or accumulated in root vacuoles (combined with organic acids or chelated by phenolic compounds). Transport into shoots of divalent cations like Fe, Ca, Mg and Cu was also inhibited by high Mn concentration in the solution (Table 6). The antagonistic effect of Mn on Ca, Mg and Fe uptake has been well documented (Marschner 1995). Leaf chlorosis may be the result of a Mn-induced Fe or Mg deficiency (Marschner 1995) or pigment photooxidation induced by an oxidative stress (Fernando and Lynch 2015; Noctor et al. 2015). When Fe supply was increased (in the form of Fe-EDDHA) in the nutrient solution, chlorosis disappeared with the improved content in photosynthetic pigments and Fe and the biomass production was improved (Table 1, Fig. 3). However, leaf Mn contents was not reduced by Fe addition as reported in other species (Zaharieva 1995). The lower chlorophyll *a* and *b* and carotenoids content at high Mn contents might result in photosynthetic inhibition as reported in other species (Li et al. 2010; Millaleo et al. 2013). Shoot growth was more sensitive to high Mn than root growth (Table 1) even though leaf Mn contents were lower (1,328-3,619 mg Mn kg^{-1}) than in roots (9,564-14,036 mg Mn kg^{-1}) (Table 6). This differential sensitivity between leaves and roots might be due to a greater root capacity for Mn sequestration into vacuoles or fixation in cellular structures either oxidized or chelated.

The excess of Mn may lead to overproduction of oxygen reactive species (ROS) by Fenton reaction on metabolically generated H_2O_2 (e.g. by mitochondrial respiration, apoplastic NADPH oxidases, cell wall peroxidases, etc.) or other metabolic process where Mn interference might induce additional oxidative stress (Noctor et al. 2015; Sharma et al. 2016; Berni et al. 2018). Phenolic compounds, carboxylates and some amino acids, may chelate or sequester the element in extra- or intracellular compartments (Mahal et al. 2005; Callahan et al. 2006; Sharma and Dietz 2006; Flis et al. 2016). High Mn concentration in the medium increased phenolic contents and amino acids (aspartate, glutamate, arginine, asparagine and glutamine) in roots (Tables 2 and 5). Phenolics may sequester the excess of metal (Baldisserotto et al. 2004) but also they are effective antioxidants avoiding cellular damage induced by reactive oxygen species (ROS) (Mahal et al. 2005; Michalak 2006). The increased phenolic synthesis is a general response under metal stress (Michalak 2006; Berni et al. 2018). Meanwhile, the increase of the glutamate cycle amino acids might correspond to the change in redox state of cells (Gulyás et al. 2917) induced by Mn excess depleting the ascorbate/glutathione antioxidative pools (Noctor et al. 2015). As a result of this metabolic re-programming, some amino acids may serve as metal ligands (e.g. asparagine, aspartic, glutamine) (Sharma and Dietz 2006; Clemens 2019) or are required (e.g. arginine) for the synthesis of antioxidants (polyamines) (Noctor et al. 2015).

High Mn concentration in the plant also induced greater activity of enzymes like superoxide dismutase in roots and catalase in leaves (Table 3) which may protect plants against oxidative stress (Noctor et al. 2015; Del Río et al. 2018). An increased activity of Mn-SOD isoenzyme may be expected at toxic Mn levels (Leidi et al. 1987; González et al, 1998; Bowler et al. 1991) at a time in which Mn stress-induced an increase in

318 mitochondrial respiration resulting in supply of organic acids (Venekamp 1989; Noctor
319 et al. 2015).

320 The critical Mn concentration in plants for toxicity is different according to
321 species and varieties and may widely vary between 200-5300 mg Mn kg^{-1} (Marschner
322 1995). In most plant species, the Mn concentration considered adequate for normal
323 growth varies from 30 to 500 mg kg^{-1} (Clarkson 1988). The Ericaceae family has an
324 extraordinary ability to bioaccumulate Mn in the leaves (Schüürmann and Markert
325 1998). Under field conditions, shoot/leaves of *E. andevalensis* accumulate more than
326 1,000 mg Mn kg^{-1} when sampled in areas with acid pH and mine-contaminated soils
327 (Abreu et al. 2008; Márquez-García and Córdoba 2010; Monaci et al. 2011) with no
328 signs of oxidative stress (Márquez-García and Córdoba 2010). A still unexplored field is
329 the possible role of root mucilages in metal binding (Morel et al. 1986) which are
330 abundantly secreted by Ericaceae roots (Leiser 1968). As pointed out above, the Mn
331 accumulation behaviour of this species may be the result of several mechanisms to
332 avoid free cellular Mn^{2+} (like cell walls sequestration, chelation and vacuolar storage)
333 and enzymatic antioxidant systems (SOD, catalase) to reduce cellular damage if ROS
334 are produced at any place by the toxicant. In the field, the Mn translocation factor value
335 indicated an opposite pattern found in our study since the leaf accumulated more Mn
336 than root (TC >1, Monaci et al. 2011; Pérez-López et al. 2014). This is a consequence
337 of the low Mn concentration in the available fraction of the mining soils (Monaci et al.
338 2011; Pérez-López et al. 2014) in spite of soil acidity. Under these conditions, *E.*
339 *andevalensis* efficiently translocates Mn into the shoot as it is an essential element.
340 Under our controlled conditions, the concentration of the available Mn was high and the
341 roots accumulated high levels of Mn. In the shoots, Mn tolerance in *E. andevalensis*
342 resulted largely because of metal root fixation, which controls translocation and plays

343 an important role avoiding metal built up in leaves (Marschner 1995; El-Jaoual and Cox
344 1998, Millaleo et al. 2010; Singh et al. 2016).

345 In comparison with Mn-hyperaccumulator species like *Acanthopanax*
346 *sciadophylloides* (Memon and Yatazawa, 1984) or *Phytolacca americana* (Dou et al.
347 2009), in *E. andevalensis* the excess of Mn was not associated with an increase in leaf
348 oxalate (Table 4). Citrate and malate in the leaves and malate and oxalate in the roots
349 were recorded as the main carboxylates whose concentration increased by high Mn
350 concentration probably related to their capacity to complex it in acid cell environments
351 like vacuoles (Flis et al. 2016; Clemens 2019). Malate and citrate have been reported to
352 be the main organic ligands for Mn stored in the vacuoles (Blamey et al. 2006; Haydon
353 and Cobbett 2007). However, the role of o carboxylates like malonate, α-cetoglutarate
354 or succinate reported in other species should not be discarded (Führs et al. 2012).

355

356 **Conclusions**

357 *Erica andevalensis* may tolerate high concentration of Mn without specific Mn
358 toxicity symptoms by restricting shoot Mn contents. Although Mn retention in the roots
359 may contribute to the Mn tolerance in this species, this mechanism was not enough to
360 avoid decrease of photosynthetic pigments and biomass production. The lower uptake
361 of essential nutrients (Ca, Cu Fe, Mg and Zn) induced by high Mn concentration in the
362 nutrient solution might be one of the factors involved in growth inhibition. However,
363 synthesis of protective compounds (phenolics, carboxylates) which may play an
364 important role as antioxidants or metal ligands might divert energy resources required
365 for growth. Also several amino acids (aspartate, glutamate, arginine, asparagine and
366 glutamine) might be involved in Mn tolerance. Manganese had an antagonist effect on
367 Fe uptake and an additional supply of Fe in the medium increased photosynthetic
368 pigments, biomass production and relieved leaf chlorosis.

References

Abreu, M.M., Tavares, M.T., Batista, M.J., 2008. Potential use of Erica andevalensis and Erica australis in phytoremediation of sulphide mine environments: São Domingos, Portugal. *Journal of Geochemical Exploration* 96, 210-222.

Aebi, H. (1984) Catalase in vitro. Methods in Enzymology 105, 121-126.

Baldisserotto, C., Ferroni, L., Meidi, V., Pagnoni, A., Pellizzari, M., Fasulo, M.P., Gafiolñi, F., Bonora, A., Pancaldi, S., 2004. Specific intra-tissue responses to manganese in the floating lamina of *Trapa natans* L. *Plant Biology* 6, 578-589.

Berni, R., Luyckx, M., Xu, X., Legay, S., Sergeant, K., Hausman, J.F., Lutts, S., Cai, G., Guerriero, G. (2018) Reactive oxygen species and heavy metal stress in plants: Impact on the cell wall and secondary metabolism. *Environmental and Experimental Botany* 161, 98-106.

Blamey, F.P.C., Hernandez-Soriano, M.C., Cheng, M., Tang, C., Paterson, D.J., Lombi, E.,Wang, W.H., Scheckel, K.G., Kopittke, P.M. (2015) Synchrotron-based techniques shed light on mechanisms of plant sensitivity and tolerance to high manganese in the root environment. *Plant Physiology* 169, 2006-2020.

Bowler, C., Slooten, L., Vandenbranden, S., De Rycke, R., Botterman, J., Sybesma, C., Van Montagu, M., Inze, D. (1991) Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. The EMBO Journal 10, 1723-1732.

Bradford, M,M, (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248-254.

Cabezudo, B., Rivera, J. (1980). Notas taxonómicas y corológicas sobre la Flora de Andalucía occidental, 2: Erica andevalensis Cabezudo y Rivera sp. nov. Lagascalia 9, 223-226

Callahan, D.L., Baker, A.J.M., Kolev, S.D., Wedd, A.G. (2006) Metal ion ligands in hyperaccumulating plants. *Journal of Biological Inorganic Chemistry* 11, 2–12. doi: 10.1007/s00775-005-0056-7.

Clarkson, D.T., 1988. The uptake and translocation of manganese by plant roots. In: Graham, R.D., Hannam, R.J., Uren, N.J. (Eds.). Manganese in soil and plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 101-111.

Clemens, S., 2019. Metal ligands in micronutrient acquisition and homeostasis. *Plant Cell and Environment* 42, 2902-2912.

Del Río, L.A., Corpas, F.J., López-Huertas, E., Palma, J.M. (2018) Plant superoxide dismutases: Function under abiotic stress conditions. In: *Antioxidants and Antioxidant Enzymes in Higher Plants* (Gupta, D.K., Palma, J.M., Corpas, F.J., eds.) pp. 1-26. Springer Int. Publ. doi.org/10.1007/978-3-319-75088-0_1

Dou, C.M., Fu, X.P., Chen, X.C., Shi, J.Y., Chen, Y.X. (2009) Accumulation and interaction of calcium and manganese in *Phytolacca americana*. Plant Science 177, 601-606.

Dou, C.M., Fu, X.P., Chen, X.C., Shi, J.Y., Chen, Y.X. (2009) Accumulation and detoxification of manganese in hyperaccumulator *Phytolacca americana*. *Plant Biology* 11, 664–670.

Ernst, W.H.O., Kerkleij, J.A.C., Shat, H. (1992) Metal tolerance in plants. *Acta Botanica Neerlandica* 41, 229-248.

Fecht-Christoffers, M.M., Führs, H., Braun, H.P., Horst, W.J. (2006) The role of hydrogen peroxide-producing and hydrogen peroxide-consuming peroxidases in the leaf apoplast of cowpea in manganese tolerance. *Plant Physiology* 140, 1451–1463.

Fernando, D.R., Lynch, J.P. (2015) Manganese phytotoxicity: new light on an old problem. *Annals of Botany* 116, 313–319.

Flis, P., Ouerdane, L., Grillet, L., Curie, C., Mari, S., Lobinski, R. (2016) Inventory of metal complexes circulating in plant fluids: a reliable method based on HPLC coupled with dual elemental and high-resolution molecular mass spectrometric detection. *The New Phytologist* 211, 1129–1141.

Foy, C.D., Chaney, R.L., White, M.C. (1978) The physiology of metal toxicity in Plants. *Annual Review of Plant Physiology* 29, 511-566.

Führs, H., Specht, A., Erban, A., Kopka, J., Horst, W.J. (2012) Functional associations between the metabolome and manganese tolerance in *Vigna unguiculata*. *Journal of Experimental Botany* 63, 329-340.

Giannopolities, C.N., Ries, S.K. (1977) Superoxide dismutases. I. Occurrence in higher plants. *Plant Physiology* 59, 309-314.

González, A., Steffen, K.L., Lynch J.P. (1998) Light and excess manganese. Implications for oxidative stress in common bean. *Plant Physiology* 118, 493–504.

Gulyás, Z., Simon-Sarkadi, L., Badicsa, E., Nováka, A., Mednyánszky, Z., Szalaia, G., Galiba, G., Kocsya, G. (2017) Redox regulation of free amino acid levels in Arabidopsis thaliana. *Physiologia Plantarum* 159, 264–276.

Heinrikson, R.L., Meredith, S.C. (1984) Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenyliso-thiocyanate. *Analytical Biochemistry* 136, 65-74.

Horiguchi, T. (1987) Mechanism of manganese toxicity and tolerance of plants. *Soil Science and Plant Nutrition* 33: 595-606. DOI: 10.1080/00380768.1987.10557608

Jiménez, A., Hernández, J.A., de1 Rio, L.A., Sevilla, F. (1997) Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant Physiology* 114, 275-284.

Kabata-Pendias, A., (2011). Trace Elements in Soils and Plants, 4rd ed. CRC Press, BocaRaton, FL.

Krämer, U. (2010) Metal hyperaccumulation in plants. *Annual Review of Plant Biology* 61, 517-534.

Haydon, M.J., Cobbett, C.S. (2007) Transporters of ligands for essential metal ions in plants. *The New Phytologist* 174, 499-506.

Kopittke, P.M., Blamey, F.P., Asher, C.J., Menzies, N.W. (2010) Trace metal toxicity in solution culture: A review. *Journal of Experimental Botany* 61, 945-954.

Leidi, E.O., Gómez, M., Del Rio, L.A. (1987) Evaluation of biochemical indicators in soybean plants. Superoxide dismutases, chlorophyll contents and photosystem II activity. *Journal of Plant Nutrition* 10, 261-271.

Leidi, E.O., Gómez, M., Del Rio, L.A. (1989) Peroxidase isozyme patterns developed by soybean genotypes in response to manganese and iron stress. *Biochimie und Physiology der Pflanzen* 85, 391 396.

Li Q, Chen LS, Jiang HX, Tang N, Yang LT, Lin ZH, Li Y, Yang GH (2010) Effects of manganese-excess on CO_2 assimilation, ribulose-1,5-bisphosphate carboxylase/ oxygenase, carbohydrates and photosynthetic electron transport of leaves, and antioxidant systems of leaves and roots in *Citrus grandis* seedlings. *BMC Plant Biology* 10, 42. doi: 10.1186/1471-2229-10-42.

Lichtenthaler, H.K. (1987) Chlorophylls and carotenoids: pigments of photosynthetic *membranes*. In: Methods in Enzymology, vol. 148 (Colowick SP, Kaplan NO, eds.) pp. 350–382. Academic Press, San Diego.

Mahal, H,S., Kapoor, S., Satpati, A.K., Mukherjee, T. (2005) Radical scavenging and catalytic activity of metal-phenolic complexes. *Journal of Physical Chemistry* B 109, 24197-24202.

Márquez-García, B., Córdoba, F. (2010) Antioxidative system in wild populations of *Erica andevalensis*. *Environmental and Experimental Botany* 68, 58–65.

Marschner, H., 1995. Mineral Nutrition of Higher Plants, Academic Press, Harcourt Brace & Company, Publishers.

Memon, A.R., Yatazawa, M. (1984) Nature of manganese complexes in manganese accumulator plant *Acanthopanax sciadophylloides*. *Journal of Plant Nutrition* 7, 961-974.

Michalak, A. (2006) Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. *Polish Journal of Environmental Studies* 15, 523-530.

Migocka, M., Papierniak, A., Maciaszczyk-Dziubińska, E., Poździk, P., Posyniak, E., Garbiec, A., Filleur, S. (2014) Cucumber metal transport protein MTP8 confers increased tolerance to manganese when expressed in yeast and *Arabidopsis thaliana*. *Journal of Experimental Botany* 65, 5367-5384.

Millaleo, R., Reyes-Díaz, M., Ivanov, A.G., Lora, M.L., Alberdi, M. (2010) Manganese as essential and toxic element for plants: transport, accumulation and resistance mechanisms. *Journal of Soil Science and Plant Nutrition* 10, 476-494.

Millaleo, R., Reyes-Díaz, M., Alberdi, M., Ivanov, A.G., Krol, M., Hüner, N.P.A. (2013) Excess manganese differentially inhibits photosystem I versus II in *Arabidopsis thaliana*. *Journal of Experimental Botany* 64, 343-354.

Monaci, F., Leidi, E.O., Mingorance, M.D., Valdés, B., Rossini Oliva, S., Bargagli, R., (2011) The selective uptake of major and trace elements in *Erica andevalensis*, an endemic species to extreme habitats in the Iberian Pyrite Belt. *Journal of Environmental Science* 23, 444-452.

Morel, J.L., Mench, M., Guckert, A. (1986) Measurement of Pb^{2+}, Cu^{2+} and Cd^{2+} binding with mucilage exudates from maize (*Zea mays* L.) roots. *Biology and Fertility of Soils* 2, 29-34.

Noctor, G., Lelarge-Trouverie, C., Mhamdi, A. (2015) The metabolomics of oxidative stress. *Phytochemistry* 112, 33-53.

Pittman, J.K. (2005) Managing the manganese: molecular mechanisms of manganese transport and homeostasis. *The New Phytologist* 167, 733-742.

Reichman, S.M. (2002) The responses of plants to metal toxicity: A review focusing on copper, manganese and zinc. Australian Minerals & Energy Environment Foundation, Melbourne, Australia. ISBN 1-876205-13-X.

Rodríguez, N., Amils, R., Jiménez-Ballesta, R., Rufo, L. (2007). Heavy metal content in *Erica andevalensis*: an endemic plant from the extreme acidic environment of Tinto River and its soils. *Arid Land Research and Management* 21, 51–65.

Rossini-Oliva, S., Mingorance, M.D., E.O. Leidi. (2012). Tolerance to high Zn in the metallophyte *Erica andevalensis* Cabezudo & Rivera. *Ecotoxicology* 21, 2012-2021.

Rossini-Oliva, S., Abreu, M. M., Leidi, E.O. (2018). A review of hazardous elements tolerance in a metallophyte model species: *Erica andevalensis*. Geoderma 319, 43-51.

511 Sharma, S.S., Dietz, K.J. (2006) The significance of amino acids and amino acid-
512 derived molecules in plant responses and adaptation to heavy metal stress. *Journal of*
513 *Experimental Botany* 57, 711-726.
514 Sharma, S.S., Dietz, K.J. (2009) The relationship between metal toxicity and cellular
515 redox imbalance. *Trends in Plant Science* 14, 43-50.
516 Sharma, S.S., Dietz, K.J., Mimura, T. (2016) Vacuolar compartmentalization as
517 indispensable component of heavy metal detoxification in plants. *Plant Cell and*
518 *Environment* 39, 1112–1126.
519 Schüürmann, G., Markert, B.A., (1998). Ecotoxicology: Ecological Fundamentals,
520 Chemical Exposure, and Biological Effects John Wiley-Interscience Series, New York,
521 USA.
522 Singh, S., Parihar, P., Singh, R,, Singh, V.P., Prasad, S.M. (2016) Heavy metal
523 tolerance in plants: Role of transcriptomics, proteomics, metabolomics, and ionomics.
524 *Frontiers Plant Science* 6, 1143. doi: 10.3389/fpls.2015.01143.
525 El-Jaoual, T., Cox, D.A. (1998) Manganese toxicity in plants. *Journal of Plant Nutrition*
526 21: 353-386.
527 Xu, X., Shi, J., Chen, X., Chen, Y., Hu, T. (2009) Chemical forms of manganese in the
528 leaves of manganese hyperaccumulator *Phytolacca acinosa* Roxb. (Phytolaccaceae).
529 *Plant Soil* 318, 197-204.
530 Yao, Y., Xu, G., Mou, D., Wang, J., Ma, J. (2012) Subcellular Mn compartation,
531 anatomic and biochemical changes of two grape varieties in response to excess
532 manganese. *Chemosphere* 89, 150–157.
533 Zaharieva, T. (1995) Iron-manganese interactions in peanut plants as influenced by the
534 source of applied iron. In: *Iron Nutrition in Soils and Plants* (Abadia J, ed.) pp. 277-
535 282. Kluwer Acad. Publ.

Zhao, J., Wang, W., Zhou, H., Wang, R., Zhang, P., Wang, H., Pan, X., Xu, J. (2017) Manganese toxicity inhibited root growth by disrupting auxin biosynthesis and transport in Arabidopsis. *Frontiers in Plant Science* 8, 272. doi: 10.3389/fpls.2017.00272

A Mucilaginous Root Sheath in Ericaceae
Andrew T. Leiser
American Journal of Botany
Vol. 55, No. 3 (Mar., 1968), pp. 391-398
Published by: Wiley
DOI: 10.2307/2440426

Regulation of cytosol acidity in plants under conditions of drought
JH Venekamp - Physiologia Plantarum, 1989 –

Pérez-López, R., Márquez-García, B., Abreu MM, Nieto JM, Córdoba F (2014) Erica andevalensis and Erica australis growing in the same extreme environments: Phytostabilization potential of mining areas. Geoderma Volumes 230–231, October 2014, Pages 194-203.

Erica andevalensis and Erica australis growing in the same extreme environments: Phytostabilization potential of mining areas